(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 August 2002 (08.08.2002)

PCT

(10) International Publication Number
WO 02/060952 A1

(51) International Patent Classification[7]: C07K 14/81, C12N 15/15, 15/63, 5/10, A61K 38/55, G01N 33/68, C07K 1/22

(21) International Application Number: PCT/GB02/00405

(22) International Filing Date: 30 January 2002 (30.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0102447.0 31 January 2001 (31.01.2001) GB

(71) Applicant *(for all designated States except US)*: **CAMBRIDGE UNIVERSITY TECHNICAL SERVICES LIMITED** [GB/GB]; The Old Schools, Trinity Lane, Cambridge, Cambridgeshire CB2 1TS (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CARRELL, Wayne, Robin** [NZ/GB]; 19 Madingley Road, Cambridge, Cambridgeshire CB3 0EG (GB). **HUNTINGTON, James, Andrew** [US/GB]; 49 Caraway Road, Fulbourn, Cambridge, Cambridgeshire CB1 5DU (GB). **ZHOU, Aiwu** [CN/GB]; 18 Manhattan Drive, Cambridge, Cambridgeshire CB4 1JL (GB).

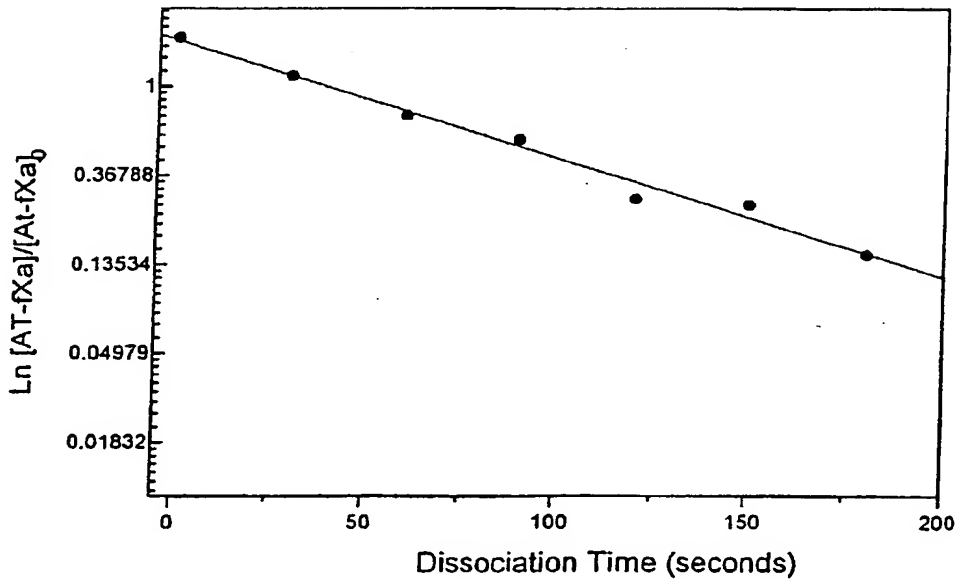
(74) Agents: **SUTCLIFFE, Nicholas, R** et al.; Mewburn Ellis, York House, 23 Kingsway, London, Greater London WC2B 6HP (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: SERPIN VARIANTS AND USES THEREOF



(57) Abstract: The present invention relates to generation and use of protein variants of serpins (SerineProteaseInhibitors). Serpins bind and inhibit serine proteases and both serpins and serine proteases are highly conserved structurally. The amino acid sequences of various different serpins may be altered, in each case creating a serpin variant, with the effect of generating a protein that forms a complex with target serine protease then releases the protease over time - intact and active. This contrasts with the natural activity of serpins which is to bind the target serine protease and irreversibly inhibit it, destroying activity and ultimately targeting the protease for destruction. Polypeptides, methods and means related to these serpin variants are provided.

WO 02/060952 A1

European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

— *with international search report*

SERPIN VARIANTS AND USES THEREOF

The present invention relates to generation and use of protein variants. In particular it relates to serpins (SerineProteaseInhibitors), proteins which bind and inhibit serine proteases. Both serpins and serine proteases are highly conserved structurally. The present inventors have shown that alterations to the amino acid sequences of various different serpins, in each case creating a serpin variant, can be made with the effect of generating a protein that forms a complex with target serine protease then releases the protease over time - intact and active. This contrasts with the natural activity of serpins which is to bind the target serine protease and irreversibly inhibit it, destroying activity and ultimately targeting the protease for destruction.

The ability to reversibly inactivate serine proteases, then "slow-release" them provides basis for a number of exciting uses and therapeutic prospects, as discussed further below. Because serpins are so highly conserved structurally, the results with one are readily repeatable with others, as is demonstrated by the various experimental examples included below.

The serpins have evolved to the predominant family of serine-protease inhibitors in man. As reported in Huntington *et al.* (2000) *Nature* 407: 923-926, the inhibition is irreversible and results from a conformational change initiated by reaction of the active serine of the protease with the reactive centre of the serpin. Deformation of the protease is brought about by the overlap in the complex of the structures of the hyperstable serpin and the destabilised protease. A principal contribution to the loss of stability in the protease is the distortion of its active site as a consequence of the ester

linkage of the catalytic serine of the protease to the reactive loop of the serpin, which then pulls it away from its catalytic partners. This distortion of the active site disrupts the interactions in the protease that were formed to stabilise it at the stage of zymogen activation. More critically the displacement of the serine from the catalytic site of the protease effectively ensures the irreversibility of inhibition by preventing hydrolysis of its acyl-linkage to the reactive loop of the serpin. The tight linkage of the two molecules and resulting overlap of their structure does not affect the hyperstable serpin, but causes a great loss of structure in the protease. Disruption of the catalytic site prevents the release of the protease from the complex, and the structural disorder allows its proteolytic destruction. Effectively the serpin crushes the protease as well as inhibiting it.

Serine proteases and serpins have been subject to a great deal of study over a number of years. Serine proteases such as thrombin, trypsin, tissue plasminogen activator and subtilisin are very important proteins biologically and medically. A number of mutants and artificially created amino acid variants of both serine proteases and serpins have been found to have particular properties that differ from those of the relevant wild-type protein, some beneficially, some detrimentally (Stein and Carrell, 1995; Owne et al., 1983).

Serpins are highly conserved structurally, and one consistently conserved feature is the reactive centre loop length in the inhibitory serpins. The loop in almost all the serpins is formed by 17 residues, from the glutamate [P17] at the base of the proximal hinge of the loop to the reactive centre [P1] residue (Huber and Carrell, 1989). This is not invariant. Although no functional inhibitory serpin has more

than 17 residues in the reactive loop - antiplasmin, C1-inhibitor and CrmA (Pickup et al, 1986) have only a 16 residue loop. The only instance of an extension of loop length, an insertion of an alanine in the antiplasmin mutant Enschede, results in a loss of inhibitory activity (Holmes et al, 1987).

The present invention is founded on work in which modified forms of serpins were created by the inventors and shown to have a remarkable new property. Instead of irreversibly inhibiting a target serine protease, destroying its enzymatic activity, the modified serpins of the invention reversibly inhibit the enzyme, binding it then slowly release it from the complex in active form. In some embodiments of the present invention this is achieved by means of insertion of one or more amino acids within the reactive loop. In some embodiments this is achieved by means of alteration of one or more amino acids, especially substitution of one residue for another at one or more positions within the molecule.

The experiments prove the principle of the invention for a range of serpins, emphasizing the generality of the invention.

Brief Description of the Figures

Figure 1 illustrates measurement of dissociation rate of the complex of Add-2 α_1-antitrypsin Pittsburgh and fXa by SDS-PAGE. The Add-2 variant (20uM), was mixed with 2uM fXa at a ratio of 2.5:1 at room temperature for 1min, the activity of fXa was quenched by adding 1000-fold excess the fXa inhibitor, PPACK, to stop the reassociation of free fXa with the remaining Add-2 serpin. The samples were then collected at 0s, 15s, 30s, 45s, 60s, 75s, 90s, 105s, 120s, 135s, 150s and 180s, respectively, and mixed with pre-heated SDS loading buffer at 100°C. After SDS-PAGE, the gel was silver-stained, and the density of the

complexes bands were semi-log plotted against time, with the slope giving the dissociation rate. M, molecular weight markers (78, 66, 45, 32 kDa); C-AT, reactive loop cleaved antitrypsin; AT, intact antitrypsin; AT-fXa, the complexes of antitrypsin variants with fXa.

Figure 2 shows results of experiments in which K328A and N314A variants of α_1-antitrypsin were made (Examples 5 and 6 respectively) and shown to bind and release serine protease.

The present invention in various aspects provides polypeptides which are serpin variants that reversibly inhibit serine protease. The present invention in various aspects provides serpin variants that bind and inhibit serine protease and then dissociate from the serine protease, releasing the serine protease from the complex in active form (i.e. with serine protease activity).

A serpin variant according to the present invention preferably comprises a modified reactive loop (i.e. a reactive loop which comprises one or more sequence modifications), preferably a reactive loop into which one or more amino acids has been inserted to increase the loop length. One, two or three amino acids may be inserted in accordance with preferred embodiments of the present invention.

The reactive centre loop is defined as the peptide sequence from the reactive centre P1 amino acid extending N-terminally 17 amino acids to position P17: Huber and Carrell, 1989.

Within the loop, insertion of one or more amino acids may be between any two residues. However, certain sites are preferred, including between positions P4 and P3, and between

positions P3 and P2. The positions within the reactive loop, the "P-numbering" is as defined by Schecter and Berger, 1967.

A serpin variant according to the present invention may contain one or more additional changes compared with the starting protein or with the wild-type or natural protein. A number of different modifications to serpins are known (both naturally occurring mutants and artificially created variants) with modified properties compared with wild-type. One or more of these properties may be retained or provided in a serpin variant according to the present invention.

Modifications conferring ability to reversibly inhibit serine protease or ability to release active serine protease from an initially formed complex need not be in the reactive loop of the serpin. The present inventors have shown that modification at other residues such as Lys 328 and Asn 314 of α_1-antitrypsin confer the desired abilities. Because of the structural conservation between serpins, analogous residues in other serpins are readily identifiable. Thus, for instance, residue Asn 314 of α_1-antitrypsin corresponds to residue Ser 314 in antichymotrypsin and Gln 319 in PAI-1 (based on the numbering of cleaved PAI-1), while residue Lys 328 α_1-antitrypsin corresponds to residue His 334 in PAI-1 (based on the numbering of cleaved PAI-1), and may be altered in accordance with standard techniques.

Any serpin may be subject to the present invention. Examples that may be employed in embodiments of the present invention include antitrypsin, antichymotrypsin, PAI-1, PAI-2, heparin cofactor II, antithrombin, thryoxine binding globulin, contrapsin, antiplasmin, angiotensin, protein Z, corticosteroid binding globulin, protein C inhibitor, glia-derived neurite promoting factor, neuroserpin and the serpin

MENT (myeloid erythroid nuclear termination stage-specific protein).

In some embodiments of the invention, the serpin is not antiplasmin.

When substituting or inserting one or more amino acid residues, the replacement or inserted residue should not result in bulky or charged side chains being buried on the inside of beta sheet A. Alanine may be used, and proline may be less preferred in some embodiments, but no amino acid is excluded for all possible embodiments.

A polypeptide according to the present invention may be isolated and/or purified (e.g. using an antibody) for instance after production by expression from encoding nucleic acid (for which see below). Thus, a polypeptide may be provided free or substantially free from contaminants. A polypeptide may be provided free or substantially free of other polypeptides. The isolated and/or purified polypeptide may be used in formulation of a composition, which may include at least one additional component, for example a pharmaceutical composition including a pharmaceutically acceptable excipient, vehicle or carrier. A composition including a polypeptide according to the invention may be used in prophylactic and/or therapeutic treatment as discussed below.

A convenient way of producing a polypeptide according to the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system. Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid according to the invention). This may conveniently be

achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Nucleic acid encoding a polypeptide of the invention is provided as a further aspect of the invention.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of contaminants. Nucleic acid may

be wholly or partially synthetic and may include genomic DNA, cDNA or RNA.

Nucleic acid may be provided as part of a replicable vector, and also provided by the present invention are a vector including nucleic acid encoding a serpin variant of t0he invention, particularly any expression vector from which the encoded polypeptide can be expressed under appropriate conditions, and a host cell containing any such vector or nucleic acid. An expression vector in this context is a nucleic acid molecule including nucleic acid encoding a polypeptide of interest and appropriate regulatory sequences for expression of the polypeptide, in an *in vitro* expression system, e.g. reticulocyte lysate, or *in vivo*, e.g. in eukaryotic cells such as COS or CHO cells or in prokaryotic cells such as *E. coli*.

A further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for in vitro introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells,

baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

The present invention also provides a complex formed between a serpin variant of the invention and a serine protease. Within the complex, the serpin variant inhibits the serine protease activity of the serine protease. Over time the serine protease is released from the complex in active form, i.e. with serine protease activity.

Such complexes are useful and may be used in therapeutic or other medical contexts. A pharmaceutical composition according to the present invention, for administration to an

individual, may comprise a serpin variant as provided and may comprise a serpin variant/serine protease complex. Administration of a complex allows for targeting of the serine protease to a desired site of action, and slow release of the enzyme. This is discussed further below.

Following production of a serpin variant by expression, its ability to bind and reversibly inhibit activity of a serine protease can be tested routinely. Formation of a complex and/or release of serine protease from a complex can be determined by SDS-PAGE and in solution, e.g. as described in the experimental examples below.

According to a further aspect of the present invention there is provided a method of making a serpin variant that binds to and reversibly inhibits serine protease activity of a serine protease, the method comprising:

producing the serpin variant by expression from encoding nucleic acid;

forming a complex of the serpin variant with a serine protease;

testing for inhibition of the serine protease activity by the serpin variant and subsequent release from the complex of the serpin protease in active form.

A positive result in the testing step (compared with suitable controls in accordance with standard practice) indicates or confirms that the serpin variant has the ability to reversibly inhibit serine protease activity in accordance with the present invention.

Such a method may optionally include isolating and/or purifying the serpin variant following its production and prior to formation of the complex.

Such a method may optionally include isolating the complex once formed.

Someone performing the method may additionally perform a prior step of providing a serpin variant by altering the amino acid sequence of the serpin variant, e.g. by substitution and/or insertion of one or more amino acids as discussed. Various different variants may be provided and tested for the desired activity, e.g. in order to identify from a range of variants one or more variants with the properties desired in accordance with the present invention. Normally, alteration of the amino acid sequence of a serpin will be made by altering the coding sequence of nucleic acid encoding a serpin. One or more nucleotides may be altered to alter one or more codons and thus the encoded amino acid(s). As mentioned elsewhere herein, and will be apparent to those skilled in the art, any suitable technique for mutagenesis, especially directed or site-specific mutagenesis, can be employed in order to change the coding sequence, and thus the encoded amino acid sequence, for a serpin.

A further aspect of the present invention provides a method of identifying or obtaining a serpin variant which reversibly inhibits a serine protease or binds to and slowly releases a serine protease in active form, the method comprising:

mutating nucleic acid encoding a serpin polypeptide to provide one or more nucleic acids with sequences encoding one or more serpin polypeptides with altered amino acid sequences ("serpin variants");

expressing the nucleic acid or nucleic acids to produce the encoded serpin variant or variants;

testing the serpin variant or variants thus produced for ability to reversibly inhibit a serine protease or bind to and slowly release a serine protease in active form.

A library or diverse population of serine variants may be produced and tested for the desired abilities.

One or more serpin variants with the desired properties may be identified or selected.

After a serpin variant of the invention has been identified or obtained it may be provided in isolated and/or purified form, it may be used as desired, and it may be formulated into a composition comprising at least one additional component, such as a pharmaceutically acceptable excipient or carrier. Nucleic acid encoding the serpin variant may be used to produce the variant for subsequent use. Such nucleic acid may, for example, be isolated from a library or diverse population initially provided and from which the serpin variant was produced and identified. Screening of libraries and cloning desired nucleic acid may involved any suitable technique at the disposal of the ordinary skilled person.

Serpins have tissue recognition sites: antithrombin binds to the wall of small blood vessels; antiplasmin and plasminogen activator inhibitor PAI-1 bind to fibrin clots. This allows for targeting for therapeutic purposes, for example in thrombolytic therapy or the treatment of any other condition in which administration of a protease, in particular a serine protease, is desirable. Administration of a serpin variant allows for it to compete with natural inhibitor at the site of action, bind to the serine protease, then slowly release the enzyme to liberate the activity. For instance, administration of PAI-1 or antiplasmin at the time of thrombolytic therapy

will target the serpin variant to the clot to form complexes with the fibrin dissolving enzyme plasmin, then slowly release the enzyme to dissolve the clot. This may be used to alleviate some difficulties with restenosis experienced with tPA therapy. Tissue plasminogen activator therapy decreases clot size, but after clearance of the serine protease often the clot returns. Slow release of active t-PA after initial bolus may be used to inhibit restenosis.

If a complex of serpin and serine protease is administered, again the serpin will naturally target to its site of action, and the serine protease will slowly be released in active form. Thus, for example serpin-protease complexes may be administered in treatment of factor-VIII resistant haemophilic crises or for slow release of fibrinolytic enzymes to prevent coronary restenosis.

Whether it is a serpin variant or complex of serpin variant and serine protease according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be any suitable route, but most likely injection, especially intravenous injection.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Since serpin variants of the invention bind tightly to serine proteases, but do not irreparably damage the protease, they are useful in isolation, identification and purification of serine proteases, and may be used in assays to determine whether or not, and if so how much of, a serine protease is present in a sample. The complex formed between a serpin variant of the invention and a serine protease is stable at pH 6. Standard affinity chromatography techniques can be used so that serine protease in a sample binds to serpin variant at this pH. Subsequently, the pH may be raised to release the serine protease (e.g to pH 8.5 or above), this release being relatively rapid and quantitative.

Thus according to a further aspect of the present invention there is provided the use of a serpin variant according to this invention for purifying of a serine protease.

A further aspect provides a method of purifying a serine protease, the method comprising

immobilising a serpin variant on a solid support (e.g. beads, chromatography column, or other surface);

contacting immobilised serpin variant with material that contains or may contain a serine protease under conditions in which the serpin and serine protease bind and form a complex (if the serine protease is present);

dissociating serine protease from any complex formed (optionally preceded by purifying any complex formed).

Serine protease may be dissociated from any complex formed by, for example, increasing pH and/or temperature.

The formation of a complex between a serpin and a serine protease is a specific binding reaction. Dissociation, e.g. upon raising of the pH to for example pH 8.5 or greater, releases from binding of a serpin according to the invention the serine protease in active form. The amount of serine protease can be quantified by means of determination of the amount of enzyme activity or amount of protein, in accordance with available techniques.

Further aspects and embodiments of the present invention will be apparent to those skilled in the art with reference to the above description and the following experimental exemplification. All documents mentioned anywhere within this specification are incorporated by reference.

Experimental Exemplification

SUMMARY

Recombinant variants of Pittsburgh (P1 Arg) α_1-antitrypsin were prepared containing 1 and 2 extra alanines at position P3-4 in the reactive loop, and also with 1, 2, 3, and 4 residues deleted from P3-6. Each of the variants was mixed with factor Xa and the formation of complex and substrate cleavage products was monitored by SDS-PAGE for 60 minutes. The addition of extra residues at P3-4 gave reversible inhibition with an initial formation of the α_1-antitrypsin-fXa complex, and then its dissociation to give the active protease and cleaved α_1-antitrypsin. The deletion of more than two residues completely converted the serpin into a substrate but the deletion of one residue resulted in less efficiency of inhibition but significantly greater stability of the formed complex (half-life 5 years versus 2.5 years for the wild-type and 6 minutes for the 1-extra-alanine recombinant.

Similar experiments were performed with PAI-1 and PAI-2, with estimated half-lives of 15 and 5 minutes respectively, for the Add-1 variants.

EXAMPLE 1

Generation of Antitrypsin variants that reversibly inhibit trypsin and slowly release active trypsin

Constructs of P1 Arg(Pittsburgh) antitrypsin (sequence disclosed in Owen et al. 1983) were prepared containing deleted and inserted residues as shown in Table 1, which illustrates the reactive loop sequences of α_1-antitrypsin, PAI-1, and PAI-2 (see Examples 2 and 3 below) with the positions

of additions and deletions in each of the engineered recombinants. The loops are homologously aligned (Huber 1989). The P notation is that of Schecter and Berger 1967.

The modifications of sequence were centred on the alanine at P4, chosen by the inventors as likely to best reflect the direct effect of loop length rather than the efficiency of loop insertion. The effects of the changes in loop length on the rates of formation and dissociation of complexes with fXa, or on conversion of the inhibitor to a substrate-form with cleavage of the reactive loop, were shown in native PAGE gels.

In each case, 3ug of antitrypsin was mixed with 1.5ug of fXa at room temperature for 5s, 15s, 30s, 1 minute, 2 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, and 60 minutes respectively. Samples were mixed with preheated SDS-loading buffer at 100°C and analysed by SDS-PAGE under reducing conditions.

Quantitative measurements of association rates, stoichiometry of inhibition and apparent second-order rate constants for inhibition are given in Table 2, and dissociation rates of complexes of the variants of a-1-antitrypsin with fXa are given in Table 3. Figure 1 illustrates how the dissociation rates of complexes of antitrypsin variants with fXa were calculated.

The gel analysis confirmed that the unmodified antitrypsin Pittsburgh is an effective inhibitor of factor Xa, with rapid formation of a complex that was then stable over the 60 minutes of incubation. There was only minor formation of the cleaved (substrate) form of the inhibitor

For variant Add-1, with an extra alanine in the reactive loop, it was shown on the relevant gel that the serpin-protease complex initially formed dissociated to release the intact protease. The incubation of an excess of the ADD-1 variant with fXa was seen to give an initial formation of the complex with the protease, followed after 30 seconds by its progressive dissociation. After 10 minutes incubation all the formed complex has dissociated to give the cleaved inhibitor and intact protease. The addition of 2 alanines to the loop, Add-2, resulted in a much diminished formation of complex which again dissociated after 30 seconds to give the release of the cleaved inhibitor and the intact protease.

Deletion of residues from the loop (Del-1, 2 & 3) affected the rate of inhibition, with the deletion of 3 residues giving complete conversion to the inactive (cleaved) substrate form. The same results as those for Del-3 were obtained with the Del-4 mutant. The Del-1 gel, however, had some stability of inhibition resulting from the shortening of the reactive loop by one residue. Although formation of the complex was more gradual as compared to the normal and Add-1 & Add-2 variants, the complex once formed remained stable over 60 minutes without apparent dissociation or release of the protease. The gel results are confirmed by the quantitative values in the tables. The conversion of the inhibitor to a substrate becomes particularly marked with the deletion of 2 or more residues (Table 2). However the most significant effect is seen in the half-lives of the formed complexes in Table 3. Whereas deletion of residues from the loop caused an increase in the stability of the complex from 2.6 to more than 4.5 years, the addition of 1 and 2 residues to the loop decreased the half-life to a few minutes or seconds.

EXAMPLE 2

Generation of PAI-1 variant that reversibly inhibits and slowly releases tPA or plasmin

PAI-1 - Plasminogen Activator Inhibitor-1 is a serpin that inhibits the serine protease tissue plasminogen activator. (The sequence of PAI-1 is disclosed in Pannekoek et al. (1986) EMBO J. 5(10) 2539-2544.)

An Add-1 variant of PAI-1 was created with an extra alanine inserted at P3 (Table 1).

The variant formed a complex with cognate protease tPA or with plasmin, with the complex in both cases then dissociating to release tPA or plasmin and cleaved PAI-1.

The half-life in both cases was approximately 15 minutes.

EXAMPLE 3

Generation of PAI-2 variant that reversibly inhibits and slowly releases tPA

PAI-2 - Plasminogen Activator Inhibitor-2 is another serpin (wholly separate from PAI-1) that inhibits the serine protease tissue plasminogen activator. (The sequence of PAI-2 is disclosed in Oye et al. (1987) J. Biol. Chem. 262 (8), 3718-3725.)

An Add-1 variant of PAI-2 was created with an extra alanine inserted at P3 (Table 1).

The variant formed a complex with cognate protease tPA, with the complex then dissociating to release tPA and cleaved PAI-2.

The half-life was about 5 minutes.

EXAMPLE 4

Generation of alpha-2 antiplasmin variant that reversibly inhibits and slowly releases tPA.

Alpha-2 antiplasmin is the major physiological inhibitor of plasmin in circulation. Alpha-2 antiplasmin also inhibits tPA.

An Add-1 variant of alpha-2 antiplasmin was created with an extra alanine inserted at P3 (The sequence of human alpha-2 antiplasmin is disclosed in Holmes et al (1987) J. Biol. Chem. 262, 1659-1664.)

The variant formed a complex with cognate protease tPA, with the complex then dissociating to release tPA and cleaved alpha-2 antiplasmin.

The half life was approximately 15 minutes.

EXAMPLE 5

Use of antitrypsin variants to reversibly inhibit other serine proteases

The variants of antitrypsin generated in Example 1 were incubated with other proteases in addition to trypsin, namely thrombin and activated protein C

Both thrombin and activated protein C gave similar results similar to those with trypsin, though with individual variations in rate. Nevertheless the effect of the lengthened loop was the same in all, with the initial formation of complex being followed by its dissociation to give concomitant release of cleaved inhibitor and intact protease.

Summary of results:

Pittsburgh antitrypsin and thrombin

	k_{off} (s^{-1})
wildtype	8.35x10-8
del-1	$2.01x10^{-8}$
add-1	$2.03x10^{-5}$
add-2	very fast (estimated t1/2 of 10 seconds by SDS-PAGE)

activated protein C

wildtype	$4.25x10^{-9}$
add-1	$1.8x10^{-7}$

EXAMPLE 6

A serpin variant that reversibly inhibits serine protease, modified outside the serpin reactive loop

An α_1-antitrypsin serpin variant was generated with Lys 328 replaced with alanine. In wild-type antitrypsin Lys 328 forms a salt bridge with the conserved Asp 194 of trypsin.

It was found that the K328A serpin variant reversibly bound to and inhibited trypsin activity. On release from the complex formed with the serpin variant, trypsin regained its enzymatic activity.

The interaction by hydrogen bonding of a residue within the serpin with the Asp 194 in the serine protease is conserved in other serpin/serine protease pairs. Accordingly, similar inhibition or breakage of the possibility of forming this link, by alteration of the relevant residue in the serpin, is likely to have the same effect on serpin function, i.e. decrease the stability of the complex and allow for reversible inhibition of cognate or target serine protease.

Results are shown in Figure 2.

EXAMPLE 7

A second serpin variant that reversibly inhibits serine protease, modified outside the serpin reactive loop

An α_1-antitrypsin serpin variant was generated with Asn 314 replaced with alanine. In wild-type antitrypsin Asn 314 hydrogen bonds to the main-chain carbonyl oxygens adjacent to the reactive centre in trypsin.

It was found that the N314A serpin variant bound to and reversibly inhibited trypsin activity, releasing active trypsin from the complex. On release from the complex formed with the serpin variant, trypsin regained its enzymatic activity.

In other serpins interaction by hydrogen bonding of a side chain to main chain carbonyle oxygens in a similar position to Asn 314 in antitrypsin will stabilise the complex. Similar inhibition or breakage of the possibility of forming this link, by alteration of the relevant residue in the serpin, is likely to have the same effect on serpin function, i.e.

decrease complex stability and allow for reversible inhibition of cognate or target serine protease.

Results are shown in Figure 2.

DISCUSSION

The unique stability of the complex that serpins form with the protease that provides the serpins with an advantage over the other families of protease inhibitors (Laskowski and Qasim 2000). Because of this, evolution has selected the serpins for the control of intra- and extra-cellular proteolytic pathways in which the release of just a few molecules of protease could be disastrous. Thus the serpins have conserved the limited loop length of 17 residues necessary to ensure distortion of the active site of the protease. The exception to this general rule is the further decrease to 16 residues in the highly specialised inhibitors - CrmA and C1-inhibitor. This is explicable by the even greater stability of the serpin-protease complex formed by the 16-residue loops (as with gel Del-1 and -2 in Table 3). This is likely to be advantageous for the crucial proteolytic pathways of apoptosis and complement activation, albeit at the cost of a decreased association rate (Table 2).

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and T4 ligase were purchased from New England Biolabs, UK and oligonucleotides were synthesised by Life Technologies Ltd., Paisley, Scotland. Expression vector pQE31 and Ni-NTA gel were from Qiagen Corp. IPTG (Isopropyl-b-D-Thiogalactopyranoside) was from Melford Laboratories Ltd., Suffolk, England. Kanamycin

sulfate was from Boehringer Mannheim GmbH, Germany. Hitrp Q-Sepharose columns were from Pharmacia, Uppsala, Sweden. Ampicillin, bovine thrombin and factor Xa (fXa) were from Sigma. Substrate S2222 and S2238 were from Chromgenix.

Construction and mutagenesis of antitrypsin expression plasmid

Human antitrypsin cDNA (Bollen et al. (1983) *DNA* 2(4), 255-264) was amplified from pWombAT by PCR and inserted into expression vector pQE31 by restriction enzyme sites, BamH1 and Hind III. The recombinant plasmid was named pQE31-AT and further mutagenesis was based on this plasmid by two-step PCR. The length of the reactive centre loop was altered as shown in Table 1. All mutations were confirmed by DNA sequencing. The recombinant antitrypsin was expressed with an MRSHHHHHH-tag at the N-terminal, which can bound to Ni-NTA agarose.

Expression and purification of recombinant protein.

The recombinant antitrypsin was purified from the soluble fraction of an *E. coli* lysate. Briefly, expression plasmids were transformed into SG13009 (pREP4) and the cells were grown in 2 litres 2TY at 37°C until OD600nm=0.8-1.0, then IPTG was added to 1mM and the culture was transferred to 30°C for a further 3hr. Cells were collected by centrifugation, resuspended in buffer A (10mM phosphate buffer, pH8.0, 0.5M NaCl, 1mM β-mecaptoethanol), and disrupted by sonication. The supernatant of the cell lysate was loaded on to a Ni-NTA column (20ml bed column) and after washing to baseline with buffer A, the bound protein was eluted as a shouldered peak with an imidazole gradient (0-0.2M). The fractions were collected, dialysed against buffer B (10mM Tris-Cl, pH8.0, 1mM EDTA, 1mM β-mercaptoethanol) and loaded onto a Hitrp-Q column (5ml). The column was then washed with a NaCl gradient (0-0.5M) in buffer B. Antitrypsin was eluted as the major peak (second peak) around 0.2M NaCl. The fractions were pooled and

protein concentrations were determined spectrophotometrically using extinction coefficient of $A280nm^{1\%}5.3$ (Pannell, 1974). The samples were snap-frozen in liquid nitrogen and stored at -70°C. All antitrypsin variants were confirmed to be in pure monomeric form by SDS and native PAGE.

Analysis of antitrypsin-fXa complex formation by SDS-PAGE

Antitrypsin (3µg) was mixed with fXa (1.5µg) at room temperature, samples were taken at variant time, mixed immediately with reduced SDS-loading buffer at 100°C and heated for a further 3min. SDS-gel electrophoresis was performed according to Laemmli (Laemmli, 1970) in a 12% gel and the protein were visualised by Coomassie Blue or silver staining.

Determination of the stoichiometry of inhibition.

Stoichiometry of inhibition (SI) values for the inhibition of fXa were determined by incubating different concentrations of antitrypsin variants with fXa at room temperature from 30 min to 4hr for different variants in PBS with 0.1% PEG 8000. The residual amidolytic activity was determined by the addition of 0.1 mM S-2222 substrate. Linear regression analysis of the decrease in fXa activity with increasing concentration of antitrypsin yielded the estimates for the stoichiometry of inhibition as the intercept on the abscissa. The SI for Add-1 antitrypsin was estimated from the SDS-PAGE by comparing the band density of cleaved and complex-formed antitrypsin between 0-2min.

Rates of Inhibition of fXa by antitrypsin variants.

The rate of inhibition of fXa by recombinant antitrypsin variants was determined at room temperature by a discontinuous assay procedure (Olson and Bjork, 1993). Briefly, under pseudo-first-order conditions, 10ul 2uM or 0.2uM antitrypsin variants were mixed with 10ul 50 nM fXa in PBS and 0.1% PEG

8000. The residual protease activity was determined at various times by diluting the reaction mixture into the assay buffer containing 0.1mM S2222. The pseudo-first-order constant, Kobs, for the reaction was obtained from the slope of a semilog plot of the residual factor Xa activity against time, and the second-order rate constant, Kapp, was determined by Kobs/$[AT_0]$, where /$[AT_0]$ is the initial antitrypsin concentration. Because of the quick dissociation of AT-A2-fXa complex, the association rate for Add-2 antitrypsin against fXa was not determined.

Dissociation of serpin-protease complex (Olson ST, 1995) Antitrypsin-fXa complexes prepared by incubating 50nM or 1 μM enzyme with a 5-10 fold molar excess of antitrypsin for 2-240 min, were dissociated by 500-fold dilution into 0.2mM substrate S2222. This level of substrate would ensure the trapping of released enzyme by substrate. Complex dissociation was continuously monitored for 10-150 min by measuring the OD change at 405nm due to the reappearance of enzyme activity. Under these experimental conditions, less than 1% substrate was converted to product and less than 3% of the complex was dissociated, so that an initial linear rate of complex dissociation was measured. Data were satisfactorily fitted by the equation describing the initial rate of enzyme generation (Jesty, 1979),

$$A_t = A_0 + V_0 t + K_{off} \times [EI]_0 \times TN \times t^2/2$$

where A_t and A_0 are the absorbance at time t and time 0, respectively. V_0 is the rate of change in OD405nm at time 0, K_{off} the first order rate constant for complex dissociation, $[E\text{-}I]_0$ is the starting concentration of complex, and TN is the turnover number for hydrolysis of substrate by enzyme under the conditions of the experiment, expressed as the rate of change in OD405nm per unit of enzyme concentration. The coefficients of the second order polynomial equation were the

fitted parameters, and were calculated from the fitted coefficient of the t term using the concentration of complex and independently measured turnover number. The complex dissociation is irreversible since the inhibitor is released from the complex in an inactive, cleaved form rather than in the native intact form, and there is no significant contribution due to residual inhibitor association during the initial rate measurement.

The dissociation rate of the antitrypsin Add-2-fXa complex was too fast to be analysed by the above method. It was analysed by SDS-PAGE. Briefly, 80ul of Add-2 (20 uM) were mixed 2uM fXa at a molar ratio of 2.5:1 at room temperature for 1min, then 1mM PPACK (small molecule inhibitor of fXa) was added to neutralise the free fXa and stop the association of fXa with excess inhibitors. Samples were taken soon after and at different time intervals, and mixed immediately with reduced SDS-loading buffer at 100°C and heated for a further 3min. SDS-gel electrophoresis was Quantity One performed as above. The density of the complex bands were determined using software (Bio-Rad Tech.) and analysed by a semilog-plot against dissociation time.

REFERENCES

1. Huber and Carrell, (1989) *Biochemistry* 28, 8951-8966

2. Pickup et al., (1986) *Proc Natl Acad Sci USA* 83, 7698-7702

3. Holmes et al., (1987) *Science* 238, 209-211

4. Schechter and Berger (1967) *Biochem Biophys Res Commun* 27, 157-162

5. Laskowski and Qasim (2000) *Biochim Biophys Acta* 1477, 324-337

6. Pannell et al., (1974) *Biochemistry* 13, 5439-5445

7. Laemmli, (1970) *Nature* 227, 680-685

8. Olson et al., (1993) *Methods Enzymol.* 222, 525-559

9. Olson et al., (1995) *J.Biol.Chem.* 270, 30007-30017

10. Jesty, (1979) *J Biol Chem* 254, 10044-10050

ACKNOWLEDGEMENT

This invention was made with the support of the US Government under grants made by the National Institute of Health. The US Government has certain rights in the invention.

TABLE 1

Antitrypsin (Pittsburgh)

	P7	P6	P5	P4			P3	P2	P1 ↓	P1'
AT-Pitts	F	L	E	A			I	P	R	S
AT-Add-1	F	L	E	A	A		I	P	R	S
AT-Add-2	F	L	E	A	A	A	I	P	R	S
AT-Del-1	F	L	E	-			I	P	R	S
AT-Del-2	F	L	E	-			-	P	R	S
AT-Del-3	F	L	-	-			-	P	R	S
AT-Del-4	F	-	-	-			-	P	R	S

PAI-1

	P7	P6	P5	P4	P3		P2	P1 ↓	P1'
wtPAI-1	A	V	I	V	S		A	R	M
P1-Add-1	A	V	I	V	S	A	A	R	M

PAI-2

wtPAI-2	G	G	V	M	T		G	R	T
P2-Add-1	G	G	V	M	T	A	G	R	T

TABLE 2

SI values and apparent second-order rate constants for inhibition fXa by reactive centre loop length mutants

	AT-pitts	AT-add-1	AT-add-2	AT-del-1	AT-del-2
K_{app} ($M^{-1}S^{-1}$)	3.15×10^4	1.63×10^4	-[a]	1.02×10^4	2.98×10^3
SI	1.15	1.2	1.18[b]	1.2	2.82
K_{app}' ($M^{-1}S^{-1}$)	3.62×10^4	1.96×10^4	-	1.22×10^4	8.40×10^3

[a] The association rate is not determined because of quick dissociation of the AT-add-2-fXa complex.

[b] The SI value was estimated from SDS-PAGE.

TABLE 3

Dissociation rate of complexes of antitrypsin variants and fXa

Complex	AT-pitts-fXa	AT-add-1-fXa	AT-add-2-fXa	AT-del-1-fXa	AT-del-2-fXa
Koff (s^{-1})	8.43×10^{-9} [a]	1.79×10^{-3} [a] 1.91×10^{-3} [b]	1.33×10^{-2} [b]	4.46×10^{-9} [a]	4.77×10^{-9} [a]
$T_{1/2}$	2.6 years	~6.2min	52 secs	4.9 years	4.6 years

[a] The dissociation rates were measure by chromogenic assay.

[b] The dissociation rates were measured from SDS-PAGE.

Claims:

1. A serpin variant polypeptide which comprises a reactive centre loop having modified amino acid sequence and which reversibly inhibits serine protease

2. A serpin variant according to claim 1 wherein one or more amino acids have been inserted into said reactive loop.

3. A serpin variant according to claim 2 wherein the one or more amino acids are inserted between positions P4 and P3, and/or between positions P3 and P2 of the reactive centre loop.

4. A serpin variant polypeptide comprising a modification at one or both positions corresponding to Lys 328 and/or Asn 314 of α_1-antitrypsin.

5. A serpin variant according to any one of the preceding claims wherein the serpin is selected from the group consisting of antitrypsin, antichymotrypsin, PAI-1, PAI-2, heparin cofactor II, antithrombin, thyroxine binding globulin, contrapsin, antiplasmin, angiotensinogen, protein Z inhibitor, corticosteroid binding globulin, protein C inhibitor and glia-derived neurite promoting factor, neuroserpin and the serpin MENT (myeloid erythroid nuclear termination stage-specific protein).

6. A nucleic acid encoding a serpin variant polypeptide according to any one of the preceding claims.

7. A vector comprising a nucleic acid according to claim 6.

8. A host cell comprising a vector according to claim 7.

9. A complex comprising a serpin variant polypeptide according to any one of claims 1 to 5 and a serine protease.

10. A pharmaceutical composition comprising a serpin variant polypeptide according to any one of claims 1 to 5 or a complex according to claim 9 and a pharmaceutically acceptable excipient.

11. A serpin variant polypeptide according to any one of claims 1 to 5 or a complex according to claim 9 for use in a method of treatment of the human or animal body.

12. Use of a serpin variant polypeptide according to any one of claims 1 to 5 or a complex according to claim 9 in the manufacture of a medicament for use in the treatment of a thrombolytic condition.

13. Use according to claim 12 wherein the thrombolytic condition is restenosis.

14. A method of making a serpin variant that binds to and reversibly inhibits serine protease activity of a serine protease, the method comprising:

producing the serpin variant by expression from encoding nucleic acid;

forming a complex of the serpin variant with a serine protease;

testing for inhibition of the serine protease activity by the serpin variant and subsequent release from the complex of the serpin protease in active form.

15. A method according to claim 14 comprising the step of isolating the serpin variant prior to formation of the complex.

16. A method according to claim 14 or claim 15 comprising isolating the complex of the serpin variant and the serine protease.

17. A method according to any one of claims 14 to 16 comprising mutating nucleic acid encoding a serpin polypeptide to provide a nucleic acid encoding a serpin variant prior to expression therefrom.

18. A method of identifying or obtaining a serpin variant which reversibly inhibits a serine protease or binds to and slowly releases a serine protease in active form, the method comprising:

mutating nucleic acid encoding a serpin polypeptide to provide one or more nucleic acids with sequences encoding one or more serpin polypeptides with altered amino acid sequences ("serpin variants");

expressing the nucleic acid or nucleic acids to produce the encoded serpin variant or variants;

testing the serpin variant or variants thus produced for ability to reversibly inhibit a serine protease or bind to and slowly release a serine protease in active form.

19. A method according to claim 18 comprising producing a library of serine variants and testing the variants of said library for one or both of said abilities.

20. A method according to claim 18 or claim 19 comprising identifying one or more serpin variants with one or both of said abilities.

21. A method according to claim 20 comprising isolating said one or more serpin variants.

22. A method according to claim 20 comprising isolating nucleic acid sequence encoding said one or more serpin variants.

23. A method according to claim 21 comprising formulating said one or more isolated serpin variants into a composition comprising at least one additional component.

24. A method of purifying a serine protease, the method comprising

immobilising a serpin variant on a solid support;

contacting immobilised serpin variant with material that contains or may contain a serine protease, under conditions in which, in the presence of the serine protease, the serpin and serine protease bind and form a complex;

dissociating serine protease from any complex formed.

25. A method according to claim 24 wherein the serine protease is dissociated from the complex by increasing pH and/or temperature.

26. A method of treating a thrombolytic condition comprising administering to an individual in need thereof a serpin variant polypeptide according to any one of claims 1-5 or a complex according to claim 9.

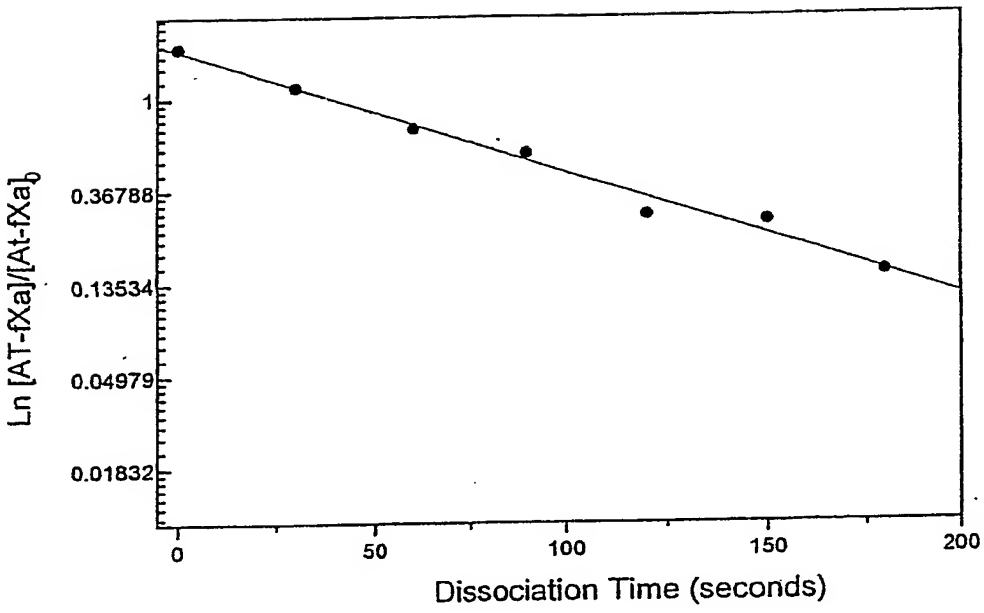

Ln [AT-fXa]/[At-fXa]0
1
0.36788
0.13534
0.04979
0.01832
0
50
100
150
200
Dissociation Time (seconds)

Figure 1

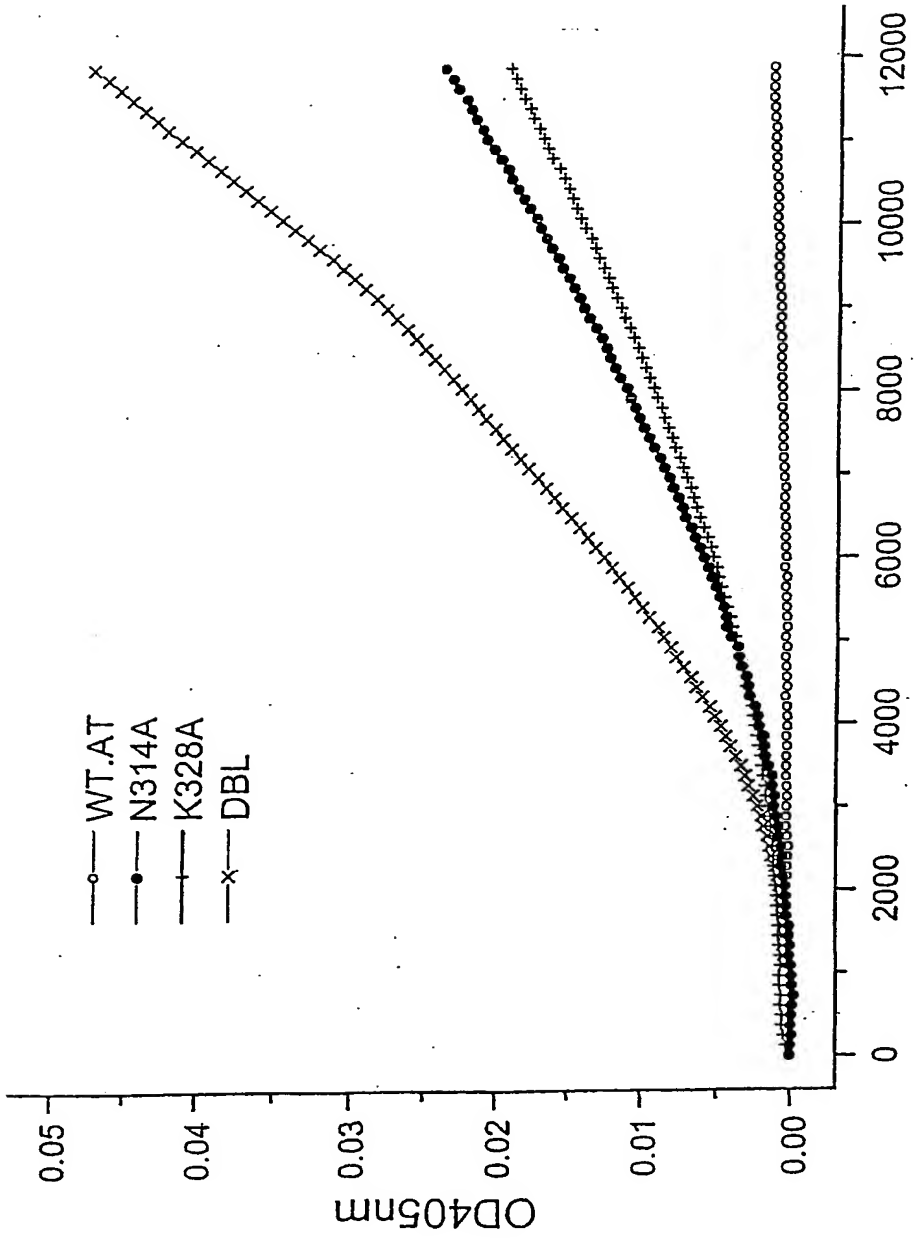

OD405nm
0.05
0.04
0.03
0.02
0.01
0.00
0
2000
4000
6000
8000
10000
12000
TIME (seconds)
WT.AT
N314A
K328A
DBL

Figure 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/00405

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/81 C12N15/15 C12N15/63 C12N5/10 A61K38/55
- G01N33/68 C07K1/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZHOU AIWU ET AL: "The serpin inhibitory mechanism is critically dependent on the length of the reactive center loop." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 29, 20 July 2001 (2001-07-20), pages 27541-27547, XP002197122 ISSN: 0021-9258 see the whole document, in particular the cited passages page 27544, right-hand column, line 26 - line 34; figures 3,5; table I page 27545, right-hand column, line 32 - line 59 --- -/--	1-3,5-26

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 2002	21/05/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel: (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Brenz Verca,' S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HUNTINGTON JAMES A ET AL: "Structure of a serpin-protease complex shows inhibition by deformation." NATURE (LONDON), vol. 407, no. 6806, 2000, pages 923-926, XP002197123 ISSN: 0028-0836 cited in the application page 925, left-hand column, line 1, paragraph 1 - line 28 page 925, right-hand column, line 15 - line 24 ---	1-26
X	RIJKEN D C ET AL: "ALPHA-2 ANTIPLASMIN ENSCHEDE IS NOT AN INHIBITOR BUT A SUBSTRATE OF PLASMIN" BIOCHEMICAL JOURNAL, vol. 255, no. 2, 1988, pages 609-616, XP002197124 ISSN: 0264-6021 page 612, right-hand column, line 1 -page 613, right-hand column, line 2; figures 4,5 page 613, right-hand column, last paragraph -page 614, left-hand column, paragraph 1 ---	1-3,5-26
X	HOLMES W E ET AL: "ALPHA-2 ANTIPLASMIN ENSCHEDE ALANINE INSERTION AND ABOLITION OF PLASMIN INHIBITORY ACTIVITY" SCIENCE (WASHINGTON D C), vol. 238, no. 4824, 1987, pages 209-211, XP001063186 ISSN: 0036-8075 page 211, left-hand column, last paragraph -middle column, paragraph 1; figure 3 ---	1-3,5-26
X	STRATIKOS EFSTRATIOS ET AL: "Formation of the covalent serpin-proteinase complex involves translocation of the proteinase by more than 70 ANG and full insertion of the reactive center loop into beta-sheet A." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 9, 27 April 1999 (1999-04-27), pages 4808-4813, XP002197125 April 27, 1999 ISSN: 0027-8424 see variant modified at position 314 table 1 --- -/--	4,6-9

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AVRON A ET AL: "EFFECT OF ALANINE INSERTION P'-5 ON THE REACTIVE CENTER OF ALPHA-1 ANTITRYPSIN" FEBS (FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES) LETTERS, vol. 280, no. 1, 1991, pages 41-43, XP001068830 ISSN: 0014-5793 page 42, left-hand column, line 20 - line 28; table I	1,6-9, 14-22
X	CHAILLAN-HUNTINGTON CATHERINE E ET AL: "The P-6-P-2 region of serpins is critical for proteinase inhibition and complex stability." BIOCHEMISTRY, vol. 36, no. 31, 1997, pages 9562-9570, XP002197126 ISSN: 0006-2960 see materials & methods section; page 9566, right-hand column, line 26 - line 30; figure 2; table 1 page 9567, left-hand column, line 5 - line 15 page 9569, right-hand column, line 41 - line 46 page 9570, left-hand column, last paragraph	1,6-9, 14-22
X	PLOTNICK MICHAEL I ET AL: "Role of the P6-P3' region of the serpin reactive loop in the formation and breakdown of the inhibitory complex." BIOCHEMISTRY, vol. 36, no. 47, 25 November 1997 (1997-11-25), pages 14601-14608, XP002197127 ISSN: 0006-2960 materials & methods section; page 14604, right-hand column, line 29 - line 35; figures 1,2A; tables 1-3 page 14607, left-hand column, paragraphs 3,4	1,6-9, 14-22

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUBIN H ET AL: "CONVERSION OF ALPHA1-ANTICHYMOTRYPSIN INTO A HUMAN NEUTROPHIL ELASTASE INHIBITOR: DEMONSTRATION OF VARIANTS WITH DIFFERENT ASSOCIATION RATE CONSTANTS, STOICHIOMETRIES OF INHIBITION, AND COMPLEX STABILITIES" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 33, no. 24, 1994, pages 7627-7633, XP002035668 ISSN: 0006-2960 page 7629, right-hand column, last paragraph -page 631, left-hand column, paragraph 1; figures 4,5; table 2 ---	1,6-9, 14-22
X	BOTTOMLEY STEPHEN P ET AL: "Protein engineering of chimeric Serpins: An investigation into effects of the serpin scaffold and reactive centre loop length." PROTEIN ENGINEERING, vol. 11, no. 12, December 1998 (1998-12), pages 1243-1247, XP002197128 ISSN: 0269-2139 chimera AT/Serp1 with fXa page 1245, left-hand column, last paragraph -page 1246, left-hand column, paragraph 1; figure 1; tables I,II,III ---	1,6-9, 14-22
X	SOKOLOVA ELENA A ET AL: "A serine protease from the bovine duodenal mucosa, chymotrypsin-like duodenase." EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 255, no. 2, 2 July 1998 (1998-07-02), pages 501-507, XP002197129 ISSN: 0014-2956 page 502, left-hand column, line 29 - line 35 ---	24
X	SCHWARTZ BRADFORD S ET AL: "Two distinct urokinase-serpin interactions regulate the initiation of cell surface-associated plasminogen activation." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 21, 21 May 1999 (1999-05-21), pages 15278-15283, XP002197130 ISSN: 0021-9258 page 15278, right-hand column, last paragraph page 15279, right-hand column, line 3 -page 15280, left-hand column, paragraph 2; figure 1 page 15280, right-hand column, line 25 - line 26; figure 3 ---	14-16

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DJIE MARYLYN Z ET AL: "Intrinsic specificity of the reactive site loop of alpha-1-antitrypsin, alpha-1-antichymotrypsin, antithrombin III, and protease nexin I." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 26, 1997, pages 16268-16273, XP002197131 ISSN: 0021-9258 page 16270, left-hand column, line 11 - line 18; tables I,II page 16271, left-hand column, last line -right-hand column, line 25	1-26
T	PLOTNICK MICHAEL I ET AL: "Heterogeneity in serpin-protease complexes as demonstrated by differences in the mechanism of complex breakdown." BIOCHEMISTRY, vol. 41, no. 1, 8 January 2002 (2002-01-08), pages 334-342, XP002197132 January 8, 2002 ISSN: 0006-2960 page 335, left-hand column, line 9 - line 15 page 340, left-hand column, line 5 - line 12	1-26

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3, 5-26 (partial)

Serpin variant which reversibly inhibit serine protease, obtained by insertion of one or more amino acids into the reactive-centre loop; nucleic acid encoding such variant; vector comprising said nucleic acid; host cell comprising said vector; complex comprising such a serpin variant and a serine protease; pharmaceutical composition comprising such a variant; first and second medical use of such a variant; method of making said serpin variant; method of identifying said serpin variant; method of purifying a serine protease involving the use of said serpin variant; method of treatment of a thrombolytic condition comprising the administration of said serpin variant.

2. Claims: 4, 5-26 (partial)

Serpin variant which reversibly inhibit serine protease, obtained by mutation of amino acids involved in interactions stabilizing the serpin-protease complex, corresponding to Lys328 and/or Asn314 in alpha-1 antitrypsin; nucleic acid encoding such variant; vector comprising said nucleic acid; host cell comprising said vector; complex comprising such a serpin variant and a serine protease; pharmaceutical composition comprising such a variant; first and second medical use of such a variant; method of making said serpin variant; method of identifying said serpin variant; method of purifying a serine protease involving the use of said serpin variant; method of treatment of a thrombolytic condition comprising the administration of said serpin variant.